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# EFFECTS OF BIOSYNTHETIC HUMAN EPIDERMAL GROWTH FACTOR ON WOUND HEALING

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## FORWARD

For the protection of human subjects the investigator(s) have adhered to policies of applicable Federal Law 45CFR46.

In conducting the research described in this report, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. (NIH) 86-23, Revised 1985).

## STATEMENT OF PROBLEM UNDER STUDY

The ability of physicians to accelerate healing of mid-dermal injuries and incisions is limited at present to preventing infections and to providing proper nutritional support. Much of the mortality, morbidity, and cost of healing major injuries such as burns or extensive surgery is closely related to the length of time required for healing of such injuries. The major goals of this contract are to evaluate the actions of biosynthetic peptide growth factors on healing of mid-dermal injuries and incisions in animals, and to test formulations of the growth factors on paired mid-dermal injuries in patients.

## BACKGROUND AND REVIEW OF APPROPRIATE LITERATURE AND EARLIER REPORT

Patients with extensive second and third degree burns are at high risk for developing life-threatening infections and other complications until the epidermal layer of their skin has regenerated. The definitive coverage of full-thickness burns is excision and autografting with split-thickness skin. Cadaver skin, pig skin, or placenta are all useful for temporary coverage of full-thickness injuries until adequate autographs can be obtained. But in severely burned patients, suitable donor sites are often the limiting factor in covering patient's burns. Substitutes for autologous skin graphs such as sheets of cultured epidermal keratinocytes or synthetic dermis containing patient's fibroblasts have shown promise but are not yet a reality and require extensive technical support facilities. Thus, there is a need for a simple agent that would accelerate the rate of epidermal regeneration of donor sites which could be reharvested at shorter intervals.

Previous reports indicated that epidermal growth factor (EGF), a small polypeptide growth factor found in human urine and blood, was a powerful mitogen for most ectodermal and mesodermal cells (1), and that EGF stimulated mitosis of keratinocytes in culture (2). Prior to our report (3), several studies had reported that topical applications of EGF applied as a mist in saline failed to stimulate healing of epidermal injuries in rats (4,5), or man (6). We reported that EGF formulated in a water-miscible cream significantly accelerated the rate of epidermal regeneration by cutting the time of healing in half for mid-dermal incisions or thermal injuries compared to injuries treated with vehicle no treatment (3). We speculated that the reason for the failures of previous studies to detect an acceleration of epidermal regeneration was due to the manner in which they applied EGF. Tissue culture studies with fibroblasts had shown that continuous exposure of cells to EGF for approximately 12 hours was required for stimulation of mitosis (7,8). Repeated topical application of EGF in a water-miscible cream maintained exposure of epidermal cells to EGF and prevented desiccation of the injury.

Healing of surgical or traumatic incisions is a major factor in the time and cost required for total rehabilitation of patients. At present, no pharmacological agent is available clinically to accelerate healing of incisions over that obtained by good surgical skill, minimizing infection and providing adequate nutrition.

Experiments performed with wound chambers in animals suggested that repeated injections of EGF or other peptide growth factors increased parameters associated with tensile strength such as content of collagen and DNA (9,10). Thus, treatment of incisions with growth factors may stimulate healing.

## RATIONAL USED IN CURRENT STUDY

Based on the ability of EGF to stimulate the rate of epidermal regeneration in mid-dermal injuries in pigs (3), we evaluated other biosynthetic peptide growth factors which might have activity on keratinocytes using the same pig regeneration model. The rational for selecting a synthetic hybrid molecule composed of the N-terminal half of transforming growth factor alpha (TGF-$\alpha$) and the C-terminal half of vaccinia growth factor (VGF) was that it is a member of the EGF family of peptides and bound to the same receptor as EGF. Other factors included , basic fibroblast growth factor (FGF), insulin-like growth factor I (IGF-I), and transforming growth factor beta (TGF-$\beta$).

In addition, we also performed a double blind clinical trial using EGF on paired donor sites. The rational for selecting these injuries for the clinical trial was because they provided uniform mid-dermal injuries in contrast to variable injuries produced by accidents. Second, donor sites could be selected on patients which were not likely to have impaired wound healing such as diabetics or patients receiving steroids or chemotherapy. Third, paired donor sites allowed for direct comparison of the rate of healing between EGF-treated and vehicle-treated injuries which would control for differences in natural healing rates between different patients.

Since we had shown that EGF in liposomes increased tensile strength of incisions during the early phases of healing (11), we tested another growth factor, TGF-$\beta$, which had the advantage that it might not need prolonged exposure time to induce biological response. Also, TGF-$\beta$ has been reported to have both stimulatory and inhibitory actions on cells (12).

We also conducted preliminary studies on the effects of EGF on healing of tympanic membrane (TM) perforations. The rational for these studies is that the histological structure of TM is very similar to that of skin with a stratified squamous epithelium covering a stromal layer of fibroblasts and an inner layer of mucoepithelium. Thus, cells of the TM may be targets for EGF action. Perforations to TM can be caused by overpressures produced by explosions in combat, and until the perforations heal, hearing is compromised and the soldier is at greatly increased risk for infections in the inner ear which can lead to permanent hearing loss.

## EXPERIMENTAL METHODS

EPIDERMAL REGENERATION. Experiments were performed as described by Brown et al. (3). Briefly, donor sites were made on the backs of pigs with a dermatome then treated topically once daily with an occlusive release dressing soaked with saline containing the test factor. Paired control donor sites were received dressings soaked with saline. At appropriate times after injury, dressings were removed, donor sites photographed, and healing evaluated by planimetry of the photographs.

CLINICAL TRIAL WITH EGF ON DONOR SITES. Patients who required split-thickness grafting for various reasons were enrolled in a prospective, randomized, double-blind trial. Each patient had two donor sites of 5 cm x 15 cm created with a Padgett dermatome set at a depth of 12/1000 inch. Wounds were treated topically twice daily with 0.5 ml/cm$^2$ of either Silvadene cream containing 10$\mu$ g hEGF per ml or Silvadene alone. Wounds were photographed daily and prints were analyzed by computer planimetry to determine the percentage of epithelialization of each wound. On day 5 post injury, 3 mm punch biopsies were obtained from each wound for histological analysis.

CUTANEOUS INCISION MODEL. Linear incisions were made through the dorsal skin of rats, test solutions were added and tensile strength measured at various times as described by Mustoe et al. (11).

TYMPANIC MEMBRANE PERFORATION MODEL. Cats underwent bilateral meatoplasties and bilateral, total perforations of the tympanic membranes (TM). Ears were treated with EGF in various formulations including saline or hydroxyproplymethyl cellulose. At appropriate times after injury, TMs were fixed in situ with formaldehyde, dissected from the bony annulus, photographed and the area of remaining perforations measured by planimetry.

## RESULTS

EPIDERMAL REGENERATION. As shown in Figure 1, EGF substantially increased the rate of epidermal regeneration during the early phases of healing compared to control injuries treated with occlusive dressing alone. Another major result was the inhibition of epidermal regeneration observed with TGF-$\beta$ treatment. Combinations of growth factors (EGF, IGF-I, FGF, TGF-$\beta$) did not improve the response in this initial experiment.

Healing of mid-dermal burns with a synthetic hybrid molecule of TGF-$\alpha$/VGF also was accelerated. At 7 days following injury all three burns treated twice daily with 1 ml of TGF-$\alpha$/VGF hybrid at 10 $\mu$g/ml in Silvadene were significantly ($p<0.05$, paired T-test) more healed than the paired burns treated with Silvadene vehicle. Since the availability of the hybrid growth factor is low, we have not pursued additional studies with the molecule.

CLINICAL TRIAL WITH EGF ON DONOR SITES. Twelve patients were enrolled in the study at Emory University. As shown in Table 1, donor sites treated with EGF healed at a significantly ($p,0.05$, paired t-test) faster rate than paired donor sites treated with vehicle. Morphometric analysis of histological specimens prepared from the punch biopsies also revealed significantly ($p<0.05$) more epithelium present on the EGF-treated sites compared to vehicle-treated sites.

TABLE 1

Clinical Trial of hEGF on Paired Donor Sites. Twelve patients received paired 5cm x 15cm donor sites created with a Padgett dermatome set at a depth of 12/1000 of an inch. Wounds were treated twice daily with 0.5 ml/cm of Silvadene cream containing 10 $\mu$g hEGF per ml or Silvadene cream alone. Wounds were photographed daily and prints were analyzed for healing by computerized planimetry.

| treatment | days to complete healing (mean) |
|---|---|
| Silvadene | 10.75 |
| Silvadene + EGF | 8.10 |

CUTANEOUS INCISIONS. As shown in Figure 2, a single treatment of incisions with a single dose of TGF-$\beta$ in collagen vehicle significantly ($p<0.05$) increased tensile strength compared to vehicle-treated control incisions at an early stage (10 days) of wound healing. At a later stage of healing (14 days), tensile strength levels of incisions treated with TGF-$\beta$ or vehicle were equal.

TYMPANIC MEMBRANE PERFORATIONS. As part of an initial study on the potential usefulness of EGF in stimulating healing of TM perforations, we determined if TM is a target tissue for EGF. Using $^{125}$I-EGF, we performed binding studies on porcine TMs and found that TMs expressed substantial levels of specific, high affinity receptors for EGF (Figure 3). Using autoradiography of $^{125}$I-EGF binding to intact TMs, we localized EGF receptors to all three cell layers of the TM with the highest level of receptors found in the stratified epithelial layer. Treatment of TM perforations in cats with EGF in saline or hydroxypropylmethyl cellulose vehicles demonstrated that EGF induced extensive hyperplasia in the stromal and epithelial layers. EGF treatment also caused faster closure of TM perforations than in contralateral TMs treated with vehicle in a 3 of 4 cats (Figure 4).

## DISCUSSION AND CONCLUSIONS

The results of the epidermal regeneration experiments demonstrate that not all growth factors act to stimulate healing. TGF-$\beta$ in particular may inhibit epidermal regeneration, and IGF-I and bFGF may be relative ineffective in comparison to EGF. Additional experimentation needs to be conducted to unequivocally establish the effects of these new growth factors. For example, different doses and different vehicles should be evaluated and combinations of growth factors should be evaluated more extensively.

If topical application of TGF-$\beta$ can be shown to consistently and substantially retard epidermal regeneration, it suggests that mid-dermal injuries that heal slowly may be a result of excess local production of TGF-$\beta$. New therapeutic concepts could be based on interfering with the actions of TGF-$\beta$ by antibodies that neutralize TGF-$\beta$ or by antibodies that block binding of TGF-$\beta$ to its receptor with out activating the receptor.

The results of the clinical trial clearly demonstrate that healing of mid-dermal injuries is accelerated by topical treatment with EGF. It is important to recognize that the clinical trial utilized normal, noncompromised donor site injuries. Thus, EGF treatment accelerated healing in normal tissue. Preliminary results with four patients with chronic, nonhealing ulcers treated with EGF are very encouraging. All four ulcers healed with EGF treatment in less than three weeks. A controlled trial with EGF in diabetic ulcers is just beginning at the University of Louisville and at other universities.

We had previously shown that treatment of incisions with EGF in liposomes significantly increased tensile strength during the early phase of healing. Treatment of incisions with a single application of TGF-$\beta$ in a soluble collagen vehicle also significantly increased tensile strength of paired incisions. The ability to use a single application of TGF-$\beta$ in a simple vehicle, i.e. soluble collagen, raises some important points. TGF-$\beta$ may be much easier to adapt to clinical use than EGF because of the simpler vehicle (soluble collagen verses liposomes). Also, TGF-$\beta$ may not require prolonged, continuous exposure to cells to induce a biological response important for incisional healing.

TGF-$\beta$ has been reported to both stimulate and inhibit cell mitosis leading to the concept that TGF-$\beta$ is a bifunctional regulator of cell growth in vitro (12). Our in vivo results with TGF-$\beta$ also show an apparent bifunctional response with stimulation of incisional healing but an inhibition of epidermal regeneration donor sites. Obviously, there is a need for more research to be done to fully understand how TGF-$\beta$ regulates healing in these two different injuries. It is possible that the major mechanism of action of TGF-$\beta$ in incisions is indirect with TGF-$\beta$ acting as a chemoattractant for macrophages which influence healing by releasing peptide growth factors in the area of an incision. In epidermal regeneration, TGF-$\beta$ may act by directly inhibiting keratinocyte mitosis in vivo as has been reported for human keratinocytes in vitro (13).

TM have not been investigated previously for interaction with growth factors. Our data clearly demonstrates that TM is a target tissue for EGF, and that topical treatment of TM perforations with EGF induced substantial hyperplasia of the epithelial and stromal layers and produced more rapid closure of the perforations. More thorough research needs to be conducted on the ability of EGF or other growth factors to speed healing of TM perforations and allow faster rehabilitation of hearing.

In summary, the concept of using exogenous growth factors to accelerate healing of mid-dermal injuries, incisions and TM perforations continues to be viable. Data from the first clinical trial of EGF in epidermal regeneration supports the data gathered from the pig model and validates the hypothesis for epidermal regeneration. Additional clinical trials are needed to evaluate the effect of EGF treatment of chronic ulcers. Results of EGF and TGF-$\beta$ treatment of incisions and TM perforations in animal models holds promise for clinical trials with growth factors once adequate preclinical studies are completed.

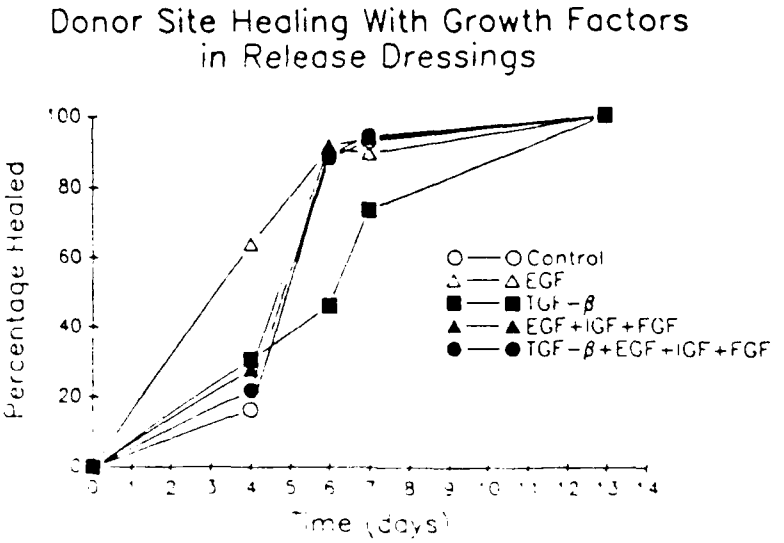


Figure 1. Donor Site Healing with Growth Factors and Release Dressings. Paired donor sites were made on the backs of pigs with a dermatome then treated topically once daily with an occlusive release dressing soaked with saline vehicle both with and without test growth factors. At the indicated times after injury dressings were removed, donor sites were photographed, and healing evaluated by planimetry of the photographs. Values are the mean of 3 to 6 donor sites. Factors were tested at 10 μg per ml.

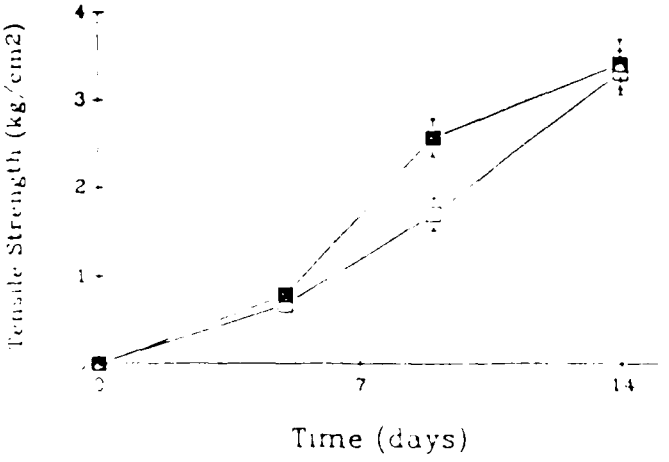


Figure 2. Tensile Strength of Incisions Treated with TGF-β. Paired linear incisions were made through the dorsal skin of rats, soluble collagen with or without 2 μg of TGF-β were added at the base of the incision which was closed with 5 interrupted sutures and at the indicated times, incisions were tested for tensile strength. Values are the mean and standard error for 9 measurements.

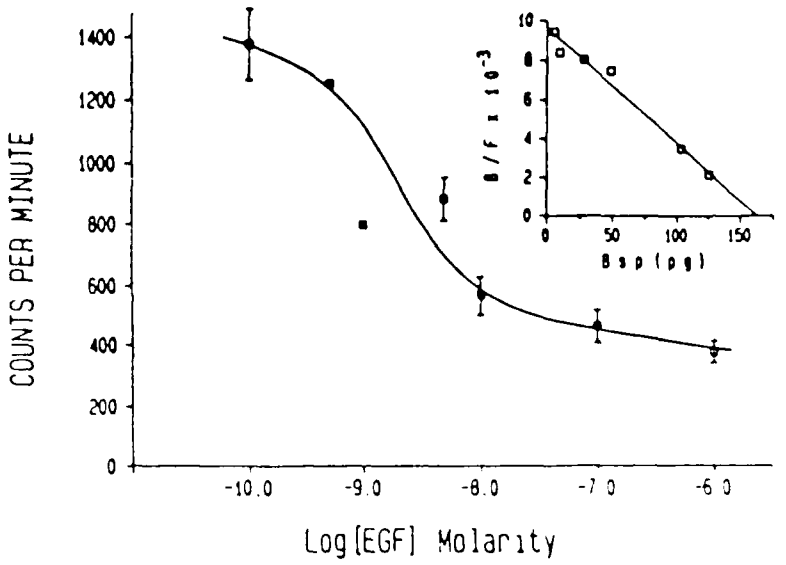


Figure 3. Characterization of EGF Binding to TM. Intact porcineTMs were incubated in CDM at 37°C for 2 hours containing $^{125}$I-EGF (100 pM) and increasing concentrations of unlabeled EGF (10 pM to 1 $\mu$M). Specific binding was transformed by method of Scatchard (insert).

Figure 4. Gross Morphology of Perforated Cat TMs Treated with EGF Impregnated Gel-foam. Total perforations of cat TMs were dosed once with Gel-foam impregnated with EGF (50 $\mu$g, left) or saline (right) at the time of surgery then fixed in situ six days later.

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19 ABSTRACT (Continue on reverse if necessary and identify by block number) The ability of physicians to stimulate healing of mid-dermal injuries and incisions in the skin is mainly limited at present to preventing infection and providing proper apposition of wound margins. The major goals of this contract are to evaluate the actions of biosynthetic peptide growth factors on stimulating healing of mid-dermal injuries and incisions to the skin. We conducted a prospective, randomized, double-blind clinical study on the effects of biosynthetic epidermal growth factor (EGF) on paired donor sites. EGF treatment significantly ($p<0.003$) accelerated healing compared to paired donor sites treated with Silvidene vehicle and decreased time to complete healing by approximately 2 days. We also found that EGF treatment stimulated healing of perforations in tympanic membrane. Testing another growth factor, we found that a single application of transforming growth factor beta (TGF-b) increased the tensile strength of surgical incisions in rats during the early period of healing. These results demonstrate that peptide growth factors can accelerate healing of several types of wounds and indicate the need for further investigations on the effects of growth factors on other wounds such as chronic ulcers and expanded mesh autografts.

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